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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: VESPER1

In re Application of:

S.J. VESPER

Serial No.: 09/866,793

Filed: May 30, 2001

For: METHODS FOR ISOLATING AND USING FUNGAL HEMOLYSINS

Art Unit: 1645

Examiner: SHAHNAN-SHAH

Confirmation No. 5682

Washington D.C.

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DECLARATION OF STEPHEN VESPER

I, Stephen Vesper, do declare that I am an inventor of the above-identified application. In order to demonstrate that extrapolating information in the specification as filed for *Stachybotrys chartarum* to other fungi, experiments were conducted under my direction and control to demonstrate that it is easy to determine which fungi produce hemolysin, and that it is easy to obtain hemolysin from a variety of fungi.

As demonstrated by the results shown in Table 1, one skilled in the art can readily determine if a fungus exhibits hemolytic activity.

In re Appl. No. 09/666,793
Confirmation No. 5682

Table 1. Observations of hemolytic activity by indoor fungi. In each case, 10 µl of each conidial suspension was plated on sheep's blood agar and incubated at 37 and 23°C. The "asterisks" represent amount of growth. One asterisk is small amount of growth, two is moderate growth and three represents good growth. If there is no growth then there is no hemolysin. To ensure that the conidia were alive they were all grown at 23°C on SBA. (Only in a few cases were hemolysins produced at 23°C.)

Fungal species	EPA #	Growth Hemolysin at		Growth Hemolysin at	
		37°C	37°C	23°C	23°C
Absidia corymbifera	133	***	No	***	No
Acremonium strictum	547	No	-	**	No
Alternaria alternata	628	*	No	**	No
Aspergillus africomus	332	No	-	**	No
Aspergillus caespitosus	371	*	No	***	No
Aspergillus versicolor	524	*	Yes	*	No
Aspergillus candidus	329	No	-	*	No
Aspergillus carbonarius	343	*	No	**	No
Aspergillus cervinus	351	No	-	*	No
Aspergillus clavatus	350	*	No	**	No
Aspergillus flavipes	610	*	Yes	**	No
Aspergillus flavus	532	***	Yes	**	No
Aspergillus fumigatus	526	***	Yes	**	No
Aspergillus niveus	361	**	Yes	**	No
Aspergillus niger	88	***	Yes	***	Yes
Aspergillus ochraceus	426	*	Yes	**	No
Aspergillus paradoxus	373	No	-	**	No
Aspergillus parasiticus	525	***	Yes	**	No
Aspergillus puniceus	368	No	-	**	No
Aspergillus restrictus	458	No	-	*	No
Aspergillus sclerotiorum	237	*	Yes	**	No
Aspergillus sydowii	421	**	Yes	**	No
Aspergillus tamarii	607	***	No	***	No
Aspergillus terreus	231	***	Yes	**	No
Aspergillus unguis	364	**	Yes	**	No
Aspergillus ustus	427	*	No	**	No
Aspergillus versicolor	524	*	Yes	*	No
Aspergillus wentii	608	No	-	**	Yes
Aureobasidium pullulans	701	No	-	*	No
Chaetomium globosum	396	**	No	***	No
Cladosporium cladosporioides	178	No	-	*	No
Cladosporium cladosporioides	174	No	-	*	No
Cladosporium herbarum	69	No	-	*	No
Cladosporium sphaerospermum	416	No	-	*	No
Emericella nidulans	527	***	Yes	**	Yes
Emericella variecolor	684	*	Yes	**	No

In re Appl. No. 09/366,793
Confirmation No. 5682

Epicoccum nigrum	335	No	-	**	No
Eurotium chevalieri	530	*	No	*	No
Memnoni lla echinata	396	*	Yes	**	No
Myrothecium verrucaria	115	***	Yes	**	No
Mucor racemosus	138	No	-	***	No
Paecilomyces varioti	75	**	Yes	*	No
Penicillium aethiopicum	310	*	Yes	**	No
Penicillium atramentosum	601	No	-	**	No
Penicillium aurantiogriseum	441	No	-	**	No
Penicillium brevicompactum	435	No	-	**	Yes
Penicillium carescens	437	*	Yes	*	No
Penicillium chrysogenum	467	*	Yes	***	No
Penicillium citreonigrum	277	No	-	*	No
Penicillium citrinum	448	*	Yes	*	No
Penicillium coprophilum	600	No	-	**	No
Penicillium corylophilum	434	No	-	*	No
Penicillium crustosum	445	No	-	**	No
Penicillium decumbens	430	*	Yes	*	No
Penicillium digitatum	316	No	-	*	No
Penicillium expansum	54	No	-	**	No
Penicillium fellutanum	431	*	Yes	*	No
Penicillium glandicola	449	No	-	**	No
Penicillium griseofulvum	456	*	Yes	*	No
Penicillium implicatum	452	No		*	No
Penicillium islandicum	616	*	Yes	*	No
Penicillium italicum	59	No	-	*	No
Penicillium janthinellum	521	**	Yes	**	No
Paecilomyces lilacinus	548	*	Yes	**	No
Penicillium lividum	292	No	-	*	No
Penicillium melinii	451	No	-	*	No
Penicillium miczynskii	443	No	-	**	No
Penicillium olsonii	523	No	-	**	No
Penicillium oxalicum	497	***	Yes	**	No
Penicillium purpurogenum	306	*	No	*	No
Penicillium raistrickii	442	No	-	**	No
Penicillium restrictum	613	*	Yes	*	No
Penicillium roqueforti	312	No	-	**	No
Penicillium sclerotiorum	453	No	-	**	No
Penicillium simplicissimum	603	*	Yes	**	No
Penicillium spinulosum	447	No	-	**	No
Penicillium variabile	322	No	-	*	No
Penicillium verrucosum	440	No	-	**	No
Penicillium waksmanii	432	No	-	**	No
Rhizopus stolonifer	58	No	-	***	No

In re Appl. No. 09/866,793
Confirmation No. 5682

Scopulariopsis brevicaulis	408	**	Yes	**	No
Scopulariopsis brumptii	409	*	No	*	No
Scopulariopsis chartarum	176	No	-	*	No
Stachybotrys chartarum	388	**	Yes	**	No
Trichoderma asperellum	538	**	No	**	No
Trichoderma harzianum	147	*	No	***	No
Trichoderma longibrachiatum	519	***	Yes	***	No
Trichoderma viride	405	No	-	***	No
Ulocladium atrum	629	*	Yes	**	No
Ulocladium botrytis	630	*	Yes	**	No
Ulocladium chartarum	631	*	No	**	No
Wallemia sebi	419	No	-	*	No

As shown in Table 1, the screening for the production of a hemolysin is simply done by placing conidia (spores) of a given fungus on 5% sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and incubating the plates at 37°C. In a week, 92 fungi were screened for hemolysin production by looking for typical darkening then clearing of the red blood cells around the colony. This is simple, inexpensive and fast. Then all one has to do is take a fungus that produce an hemolysin and grow in TSB broth as the patent teaches. Then collect the supernatant. This is important because the homogenate (like used for asp-hemolysin) introduces a huge number of other fungal proteins whereas the supernatant is relatively free of most other fungal proteins and makes the whole purification much easier and more meaningful since the secreted form of the hemolysin is the active form. Then the hemolysin is purified using standard protein purification procedure described in the patent:

Step 1- size fractionation centrifugation; Step 2- ion chromatography; Step 3- gel filtration. Obviously there is slight variations in buffers, salt concentration, gel matrix etc. but these are accepted variations that any protein biochemist expects.

To demonstrate how easy it is to isolate other fungal hemolysins using the specified directives in the patent application, I isolated the hemolysin from *Penicillium chrsogenum* using the same steps which are growth of culture in tryptic soy broth (TSB), size fractionation centrifugation of supernatant, ion chromatography, and gel filtration.

The indoor fungus *Penicillium chrysogenum* was grown on pieces of dry wall, as described (19) and the conidia recovered. Approximately 1 x 10^5 conidia were added to 500 ml of TSB. The cultures were incubated at 23°C for 48h on an incubator shaker at 100 rpm. Then the cultures were transferred to an incubator shaker and culture for 72 h at 34°C. The fungal mass was then removed by filtering through a Whatman 541 filter paper in a Buchner funnel. The recovered filtrate was centrifuged in a Millipore Centricon plus 30 filter apparatus with a MW cut-off of 30-kDa (Millipore, Bedford, MA) following the manufacturer's instructions. The concentrate was then subjected to ion exchange

chromatography. DEAE-cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris-HCl pH 8.0 for 1 hr and then poured into a column giving a final bed of 3 x 0.5 cm. Then 0.5 ml of the concentrate was introduced on the top of the column. The bed was eluted with 5 ml of the 20 mM Tris-HCl buffer and five drop fractions were collected throughout the elution and then 10 µl of each fraction was plated on sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and hemolysis noted.

Then carboxy methyl cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris-HCl pH 8.0 for 24 h and then poured into a column giving a final bed of 3 x 0.5 cm. Then the five active fractions from the DEAE-cellulose ion chromatography were introduced on the top of the column. The bed was eluted with 5 ml of the 20 mM Tris-HCl buffer and five drop fractions were collected throughout the elution and then 10 µl of each fraction was plated on sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and hemolysis noted.

The five hemolytically active fractions from the second ion exchange chromatography were then subjected to gel filtration using Sephadex G 200 (Pharmacia, Piscataway, NJ) hydrated for 72 h in the running buffer containing 0.2 M sodium azide and poured into a chromatography column to give a final bed 0.25 by

24 cm. Five drop fractions were collected at 1.5 ml per h using a fraction collector (ISCO, Lincoln, NE). Then 10 µl of each fraction was plated on SBA and incubated at 37°C and hemolysis noted at 24 h. The five hemolytically active fractions from the first gel filtration were then subjected to gel filtration using Sephadex G 100-50 (Sigma, St. Louis, MO) hydrated for 5 days in the running buffer containing 0.4 M sodium azide, 5 mM EDTA, and 1 mM PMSF and poured into a chromatography column to give a final bed 0.5 x 14 cm. Five drop fractions were collected at 1.5 ml per h using a fraction collector (ISCO, Lincoln, NE). Then 10 µl of each fraction was plated on SBA and incubated at 37°C and hemolysis noted at 48 h. The five most hemolytically active fractions from this second gel filtration were combined and then desalted twice using the D-Salt™ Polyacrylamide 6000 desalting column (Pierce, Rockford, IL.). The final desalted solution was frozen at -80°C and lyophilized using a Spin Vac (Savant Instruments, Farmingdale, NY) resulting in a lyophilized pellet.

Electrophoresis analysis

Native protein electrophoresis and SDS-PAGE were performed using the Bio-Rad Laboratories Mini-Protean™ 3 Cell and precast 4-15% Tris-HCL gels (BioRad, Hercules, CA), as per the manufacturer's instructions.

Gels were stained with silver using the Bio-Rad Silver Stain Plus™ kit by following the manufacturer's instructions (Bio-Rad, Hercules, CA).

To determine if the chrysolysin was active, a purified preparation was divided in half and run in two separate wells in a precast 4-15% Tris-HCl gel. After native protein electrophoresis, half of the gel was stained with silver, as described above, and the other half was placed on an SBA plate and incubated at 37°C for 48 h and hemolysis process photographed.

Figure 1 shows the active purified hemolysin band from *P. chroysgenum*. All of the steps described in the patent were used with modifications that any protein chemist would anticipate.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 31 of the United States Code and that such willful false statements may jeopardize the

In re Appl. No. 09/866,793
Confirmation No. 5682

validity of the application or any patent issued thereon:

7-5-02

Stephen Vesper

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Figure 1. Purified hemoysin, chrysolysin, produced by *Penicillium chrysogenum*. Left panel shows purified band of chrysolysin in "native" gel. Center panel shows appearance of sheep's blood agar (SBA) after exposure to purified chrysolysin in "native" gel for 24 h and the right panel shows appearance of SBA after 48 h exposure (SBA incubated at 37°C).